Long non-coding RNAs responsive to blast fungus infection in rice

Lanlan Wang (llwang1985@hotmail.com)
Zhejiang Academy of Agricultural Sciences https://orcid.org/0000-0002-8762-7898
Jing-Jing Jin
Zhengzhou Tobacco Research Institute
Li-Hua Li
Zhejiang Academy of Agricultural Sciences
Shao-Hong Qu
Zhejiang Academy of Agricultural Sciences

Short communication

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Abstract

Background

Long non-coding RNAs (LncRNAs) have emerged as important regulators in many physiological processes in plant. By high-throughput RNA-sequencing, many pathogen-associated lncRNAs were mapped in various plants, and some of them were proved to be involved in plant defense responses. The rice blast disease caused by *Magnaporthe oryzae* (*M. oryzae*) is one of the most destructive diseases in rice. However, *M. oryzae*-induced lncRNAs in rice is yet to be studied.

Findings:

We investigated rice lncRNAs that were associated with the rice blast fungus. Totally 83 lncRNAs were up-regulated after blast fungus infection and 78 were down-regulated. Of them, the natural antisense transcripts (NATs) were the most abundant. The expression of some lncRNAs has similar pattern with their host genes or neighboring genes, suggesting a *cis* function of them in regulating gene transcription level. The deferentially expressed (DE) lncRNAs and genes co-expression analysis revealed some lncRNAs were associated with genes known to be involved in pathogen resistance, and these genes were enriched in terpenoid biosynthesis and defense response by Gene Ontology (GO) enrichment analysis. Interestingly, one of up-regulated DE-intronic RNA was derived from a jasmonate (JA) biosynthetic gene, lipoxygenase RLL (LOX-RLL). Levels of JAs were significantly increased after blast fungus infection. Given that JA is known to regulate blast resistance in rice, we suggested that lncRNA may be involved in JA-mediated rice resistance to blast fungus.

Conclusions

This study identified blast fungus-responsive lncRNAs in rice, which provides another layer of candidates that regulate rice and blast fungus interactions.

Findings

Rice is one of the most important foods for humanity and is widely consumed in the world. Rice blast disease caused by the fungus *M. oryzae*, one of the top 10 fungal pathogens (Dean et al. 2012), is the most devastating disease of rice. The global annual crop loss due to blast was estimated at 66 billion and is enough to feed 60 million people (Pennisi 2010).

In order to cope with pathogen infection, rice has evolved a multifaceted, sophisticated defense response to microbial pathogens carrying effectors, as well as pathogen-associated molecular patterns (PAMP) (Liu et al. 2013). The first tier of plant defense is PAMP-triggered immunity (PTI) mediated by pattern recognition receptors and occurs during pathogen attachment and the early phase of host-pathogen

interactions. Activation of PTI leads to various defense responses that include the induction of an oxidative burst, activation of mitogen-activated protein kinase (MAPK) cascades, biosynthesis of hormones, accumulation of antimicrobial compounds or enzymes, and callose deposition (involved in the fortification of cell wall); which as a result, inhibits or prevents pathogen proliferation (Mitsuhara et al. 2008, Parker et al. 2009, Shimizu et al. 2010, Bundo et al. 2015, Yang et al. 2015, Delteil et al. 2016, Urso et al. 2016). The second tier of plant defense is the effectors-triggered immunity (ETI) mediated by plant resistance (R) genes, most of which encode cytoplasmic proteins with nucleotide binding site-leucine-rich repeat (NLR) domains. So far, over 100 major last R genes have been identified and 30 of them have been molecularly cloned. ETI provides a robust defense response that is often accompanied by a hypersensitive response at the infection site. However, ETI is also race-specific and fragile.

Whole genome RNA sequencing (RNA-Seq), tilling arrays and large-scale cDNA cloning studies have revealed that transcription of eukaryotic genes is highly complex (Forrest et al. 2009). The transcriptional landscape in eukaryotes has been extensively studied using RNA-Seq and reveals the RNA molecules are transcribed ranging from protein-coding mRNAs to noncoding transcripts (Chekanova et al. 2007, Berretta et al. 2009, Ponting et al. 2009, Sanchez-Leon et al. 2012, Zhu et al. 2014). Noncoding RNAs are classified into two types, containing either short sequences (< 200 nt) or long noncoding RNAs (lncRNAs, longer than 200 nt) (Guttman et al. 2009, Cabili et al. 2011, Wang et al. 2014, Zhou et al. 2014). LncRNA can in turn be classified into long intergenic noncoding RNAs (LincRNA), natural antisense transcripts (NAT) and intronic RNAs (lncRNA) based on genome location (Ponting et al. 2009, Chen 2012, Rinn et al. 2012, Lina et al. 2013, Dogini et al. 2014). LncRNAs has been revealed to function as key regulators of diverse mechanisms in arrange of biological processes. Such as regulation of gene silencing, flowering time, reproduction, stress responses, organogenesis in roots, and photomorphogenesis in seedlings (Matzke et al. 2014, Zhang et al. 2014, Berry et al. 2015, Li et al. 2016, Wang et al. 2017). Many LncRNAs show significant changes in different organs or during stress, suggesting that they are dynamically regulated and might function in development and stress responses. However, the blast fungus infection-related lncRNAs in rice remains unknown.

To test if lncRNAs are responsive to blast fungus infection, RNAs of *M. oryzae* treated samples and control were sequenced at three timepoints, each with three replicates. More than 20 million clean reads passed the quality filters (Table S1). These reads were mapped to the rice reference genome, and 95% of the clean reads were aligned for most of the samples. The transcript assembling and expression analysis was then performed. The control and treated group in each timepoint were separated well in principal component analysis (PCA) of the coding RNAs, indicating the significant variation of transcription level after blast fungus treatment (Fig. 1A-1C). A total of 4,787 transcripts were obtained and defined as lncRNA in rice. Of them, 2,366 transcripts were lincRNAs and 2,184 transcripts were NATs, while only 237 transcripts were incRNAs (Fig. 1D). The transcriptional levels of genes and these lncRNAs were compared between blast fungus treated and control samples in each timepoints. A total of 1,670 differentially expressed genes (DEGs) and 161 differentially expressed lncRNAs (DE-lncRNAs) were identified. One lincNRA was constitutively up-regulated in all of blast fungus treated plants, but other lncRNAs only showed different expression level in a particular timepoint of treatment (Fig. 1E). Among these DE-

lncRNAs, about half were up-regulated by blast fungus infection, while the other half was down-regulated (Fig. 1F). To confirm the reliability of the RNA-seq data, six DE-lncRNAs were selected and their transcript levels were confirmed by quantitative RT-PCR (qRT-PCR). The expression of two lincRNAs, TU13913 and TU29105, were significantly higher in blast fungus treated plants compared with control plants (Fig. 2A and 2B). Two of the incRNAs, TU40741 and TU7759, were up-regulated 72 h after blast fungus treatment (Fig. 2C and 2D). TU41192 was a blast fungus-specific induced NAT, which had a high expression level 72 h after treatment (Fig. 2E), while another NAT and TU3643 was down-regulated after blast fungus treatment (Fig. 2F). The expression patterns of these lncRNAs done by qRT-PCR were similar with RNA-seq data. A total number of 161 lncRNA transcripts were characterized which showed greater than 2-fold changes ($p < 0.05$) in treated plants compared to control plants.

The functions of these DE-lncRNAs in plant and blast fungus interaction were predicted. First, some lncRNAs act as cis element to regulate the expression of nearby gene (Liu et al. 2012). To predict if the blast fungus-induced lncRNAs have cis-functions, the expression of DE-lncRNA and their nearby gene were compared. The host genes of NATs and incRNAs and the neighboring genes of lincRNAs were firstly identified. The expression levels of these genes were screened based on the fold change between treated and control samples were higher than two. Totally 34 DE-lncRNA-gene pairs were identified (Fig. 3A, 3B and 3C). Of them, most of lncRNAs and their associated genes showed the same expression trend. For example, the NAT TU3643 was down-regulated by blast fungus treatment, and its host gene, RZFP34 (RING zinc-finger protein 34), was also down-regulated (Fig. 2F; Fig. 3A). RZFP34 was known to regulate stomata opening in rice (Hsu et al. 2014). Many plant pathogens including the rice blast fungus gain entry to their host via stomata, suggesting TU3643-RZFP34 may be involved in rice and blast fungus interaction. The expression of NAT TU41192 and its host gene SAG12 (senescence-associated gene) were both up-regulated by blast fungus treatment (Fig. 2E; Fig. 3A). SAG12 negatively regulate stress-induced cell death which may also play a role in blast fungus resistance (Singh et al. 2016). A gutathione S-transferase gene, GSTU4, plays a role in plant tolerance to oxidative stresses (Sharma et al. 2014). The expression of an incRNA TU7759 and its host gene GSTU4 have similar pattern in different treatment (Fig. 3B). Interestingly, a jasmonate biosynthetic gene, lipoxygenase RLL (LOX-RLL) and its intronic RNA TU40741 were also up-regulated by blast fungus treatment.

To explore the connection between DE lncRNAs and DEGs, weighted correlation network analysis (WGCNA) was performed (Langfelder et al. 2008). The co-expressed transcripts were clustered into 7 modules (Fig. 4A). Each module was indicated by different color, and the module turquoise is the most abundant with 1,270 module members. We screened the high correlated connection in module turquoise by using weight higher than 0.4. A total of 203 coding genes and 35 lncRNAs were selected, and the co-expression network was constructed (Fig. 4B). To detect which genes were associated with these lncRNAs, the gene ontology (GO) enrichment analysis was performed. Genes involved in defense response and terpenoid metabolic processes were significantly enriched (Fig. 4C). Terpenoid, especially diterpenes, are well-known to be involved in rice resistance to blast fungus (Chen et al. 2018). The network between four lncRNAs (in Fig. 2 and module turquoise) and the known pathogen resistance-related genes were picked out (Fig. 4D). These lncRNAs were co-expressed with diterpene biosynthetic

genes, jasmonate signaling pathway genes, pathogenesis-related genes, and transcription factors (Table S2). These results suggested that DE-lncRNAs may function in rice-blast fungus interaction.

TU40741 is an intronic RNA which is derived from a jasmonate (JA) biosynthetic gene, LOX-RLL. Through the network analysis from module yellow, TU40741 was also highly co-expressed with LOX-RLL gene (Fig. 5A). LOX-RLL together with AOS (allene oxide synthase) and AOC (allene oxide cyclase) catalyze linolenic acid to 12-oxo-phytodienoic acid (OPDA), the precursor of jasmonic acid. LOX-RLL transcripts were increased in blast fungus treated plants compared with control plants by qRT-PCR analysis (Fig. 5B). Sequence analysis revealed that TU40741 is derived from the second intron of LOX-RLL and has an opposite transcriptional direction with LOX-RLL gene (Fig. 5C). JA signaling pathway is known to regulate rice resistance to blast fungus (Rakwal et al. 2000, Jwa et al. 2001, Riemann et al. 2013, Shimizu et al. 2013, Yang et al. 2013, Urso et al. 2016). The products levels of LOX-RLL were measured after blast fungus infection. The levels of OPDA were significantly increased 48 h and 72 h after blast fungus treatment (Fig. 5D). However, no significant difference was observed for jasmonic acid levels (Fig. 5E), consistent with previous study (Riemann et al. 2013). The downstream bioactive forms of jasmonate, jasmonoyl-isoleucine (JA-Ile) and jasmonoyl-valine (JA-Val) levels were both enhanced in treated plants (Fig. 5F and 5G). The terpenoids were known to function as antimicrobial phytoalexins in rice (Schmelz et al. 2015). Some of monoterpene and diterpenoid are involved in rice resistance to blast fungus (Chen et al. 2018). Interestingly, the biosynthesis of these antifungal compounds is regulated by JA signaling (Riemann et al. 2013, Chen et al. 2018). LOX-RLL has been long time identified as *M. oryzae*-induced gene in rice (Peng et al. 1994). LOX-RLL was also known as herbivore-induced LOX (HI-LOX). Silencing of HI-LOX in rice decreased herbivore-induced JA levels and made plants more susceptible to chewing herbivores (Zhou et al. 2009), suggesting the role of LOX-RLL in biotic stress induced JA signaling. These data suggested that LOX-RLL-mediated JA biosynthesis may play a role in blast fungus resistance. Some intronic RNAs are characterized to regulate the expression of their host genes (Heo et al. 2011). Thus, we inferred TU40741 may regulate the expression of LOX-RLL, which in turn mediate JA signaling pathway. Emerged evidences have showed that lncRNAs were associated with pathogen-induced JA biosynthesis. Two lncRNA, GhlncNAT-ANX2- and GhlncNAT-RLP7, regulate the expression of LOX1 and LOX2 gene in cotton after fungal *Verticillium dahliae* attack (Zhang et al. 2017). In rice, the lncRNA ALEX1 activates JA pathway and plant resistance to bacterial blast (Yu et al. 2020). Further study is required to elucidate the role of LOX-RLL and TU40741 in rice defense against blast fungus.

In conclusion, we identified blast fungus-induced lncRNAs in rice by high-throughput RNA-sequencing. By co-expression analysis, some lncRNAs were predicted to be highly correlated with pathogen resistance. Strikingly, an intronic RNA was characterized to connect with blast-fungus induced JA signaling pathway. Our results provide novel candidates for the regulation study of rice-blast fungus interaction.

Materials And Methods

Plant material and fungal inoculation

Rice (*Oryza sativa* ssp *japonica* cv Nipponbare) were surface-sterilized and transferred to MS medium. After germination, rice seedlings were transplanted into vermiculite-filled pots and kept in a growth chamber at 26 °C/24 °C under a 14 h light/10 h dark cycle with 85% humidity. The *M. oryzae* strains (TMC-1) was cultured on complete medium (Zhou et al. 2016) at 25°C for 5-6 days from storage at -20°C. Then 5×10^5 spores/mL with 0.02% Tween-20 was used as final concentration for inoculation.

When two fully expanded leaves appeared, whole plants were inoculated using spraying infection method with the spores of TMC, and the inoculated plants were kept in a dark chamber at 85% humidity and 24 °C. After 34 hpi, the plants were maintained in the growth chamber at 26 °C/24 °C in a 14 h light/12 h dark cycle with 85% humidity. Leaves were collected at 24, 48, and 72 hours post inoculation. Rice plants were treated with 0.02% Tween-20 and collected at the same time points for use as mock treatments. Six to eight plants for each treatment were used. All samples were immediately frozen in liquid nitrogen and kept at −80 °C until further use.

LncRNA sequencing

LncRNA sequencing was performed by Novogene Company (Beijing, China). In brief, the total RNA without rRNA was used to construct the strand-specific RNA libraries with an insert size of 250-300 bp. The paired-end 150 bp sequencing was then performed on an Illumina Hiseq platform. Three replicates for each treatment were performed.

Transcriptome assembly and annotations

For the RNA-seq data, all sequenced reads from each experiment were trimmed using trim galore program (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with a quality score 30, then, clean data were aligned to the rice reference genome (http://rice.plantbiology.msu.edu/) using the read aligner HIAST2(Kim et al. 2015). The transcriptome of each experiment was assembled separately using stringtie (Pertea et al. 2015), all gtf result files were merged into one with stringtie –merge. Then, we compared the assembled transcript isoforms with the rice genome reference annotation information, which represents all protein coding gene models identified by rice genome project (http://rice.plantbiology.msu.edu/). Transcripts with a length shorter than 200bp and an open reading frame (ORF) length longer than 120 aa were discarded (ORF Finder, links), in order to remove transcripts that may encode short protein, swiss-prot database were searched using blastx program with the parameter -e 1.0e-4 -S 1. The CPC (Li et al. 2014)programs were used to calculate the coding potential of the remaining transcripts. Only transcripts with both CPC scores less than 0 were used for the subsequent analysis. The remaining transcripts located in intergenic regions were identified as lincRNA candidates. If the transcripts were transcribed from the antisense strands of known genes, they will be considered as NATs candidates. For the transcripts located in intron region of known genes, they were identified as incRNA candidates. The expression level of all locus identified including lncRNAs and protein-coding genes was using TPM by stringtie(Pertea et al. 2015).

Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted from the leaf blades of rice after treatment using MiniBEST Plant RNA Extraction Kit (Takara, Janpan) including DNase I treatment according to the manufacturer's instructions. RNA samples with 260/280nm ratio between 2.0-2.2, and RNA integrity number greater than 8.0, were used for the analysis. Reverse transcription was performed using 2μg of each total RNA and oligo (dt) primers by the PrimerScript RT Master Mix (Takara, Janpan). Six to eight independent biological samples were collected and analyzed. The relative quantity of lncRNAs and genes were performed using qRT-PCR. It was performed using SYBR Premix Ex TaqTM II Kit (Takara, shiga, Janpan) on the Bio-Rad CFX96 real-time system with gene-specific primers. Primer sequences used are listed in Supplemental Table S3.

Differential expression analysis

The log-transformed TPM (Trans Per Million) value was used for transcriptional level. To get the differentially expressed genes and lncRNAs, all of the transcripts from pairwise samples were screened using the adjust p value less than 0.05 and fold change higher than 1.

Co-expression analysis

The differentially expressed genes and lncRNAs were used for co-expression analysis.

Subsequent weighted gene co-expression network analysis was conducted using the R package WGCNA (Langfelder et al. 2008). The network of each module was visualized using Cytoscape (Ono et al. 2014).

PCA analysis

The log-transformed TPM (Trans Per Million) values of all coding genes were used for principle component analysis (PCA). PCA was performed using R package factoextra.

Heatmap analysis

The value of fold change in each timepoint was used for heatmap analysis. Heatmap was performed by R package pheatmap.

GO enrichment analysis

Gene ontology (GO) analysis was performed by ClueGO (Bindea et al. 2009).

Pathway with p value less than 0.05 was showed.

JA analysis

Approximately 100mg material was used for JA analysis. Samples were ground and extracted with 800 μL of ethyl acetate containing the internal standards (10 ng of D6-JA and 10 ng of D6-JA-Ile). Extracts were evaporated and dissolved with 300 μL of 70 % methanol. All samples were analyzed by UHPLC-HESI-MS/MS as previously described (Li et al. 2017).

Abbreviations

AOS: allene oxide synthase; AOC:allene oxide cyclase; DE:deferentially expressed; ETI:effectors-triggered immunity; GSTU4:gutathione S-transferase gene; GO:Gene Ontology; HI-LOX:herbivore-induced lipoxygenase; lncRNAs:intronic RNAs; JA:jasmonate; JA-Ile:jasmonoyl-isoleucine; JA-Val:jasmonoyl-valine; JAR1:jasmonic acid resistance 1; LDMAR:LONG-DAY-SPECIFIC MALE-FERTILITY-ASSOCIATED RNA; LincRNAs:long intergenic noncoding RNAs; LncRNAs:Long non-coding RNAs; LOX-RLL:lipoxygenase RLL; NATs:natural antisense transcripts; NLR:nucleotide binding site-leucine-rich repeat; OPDA:12-oxo-phytodienoic acid; PAMP:pathogen-associated molecular patterns; PTI:PAMP-triggered immunity; PCA:principal component analysis; R:resistance; SAG12:senescence-associated gene ; WGCNA:weighted correlation network analysis;

Declarations

Availability of data and material

The raw RNA-sequencing data reported in this paper have been deposited in the Genome Sequence Archive in BIG Data Center, Beijing Institute of Genomics (BIG), Chinese Academy of Sciences, under accession numbers CRA003133

Competing interest

The authors declare they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable

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Authors’ contributions

LLW designed and performed the experiments. LLW and JJJ analyzed the RNA-seq data and generated figures and tables. LLW, LHL and SHQ wrote and revised the manuscript. All authors read and approved

the final manuscript.

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Figures

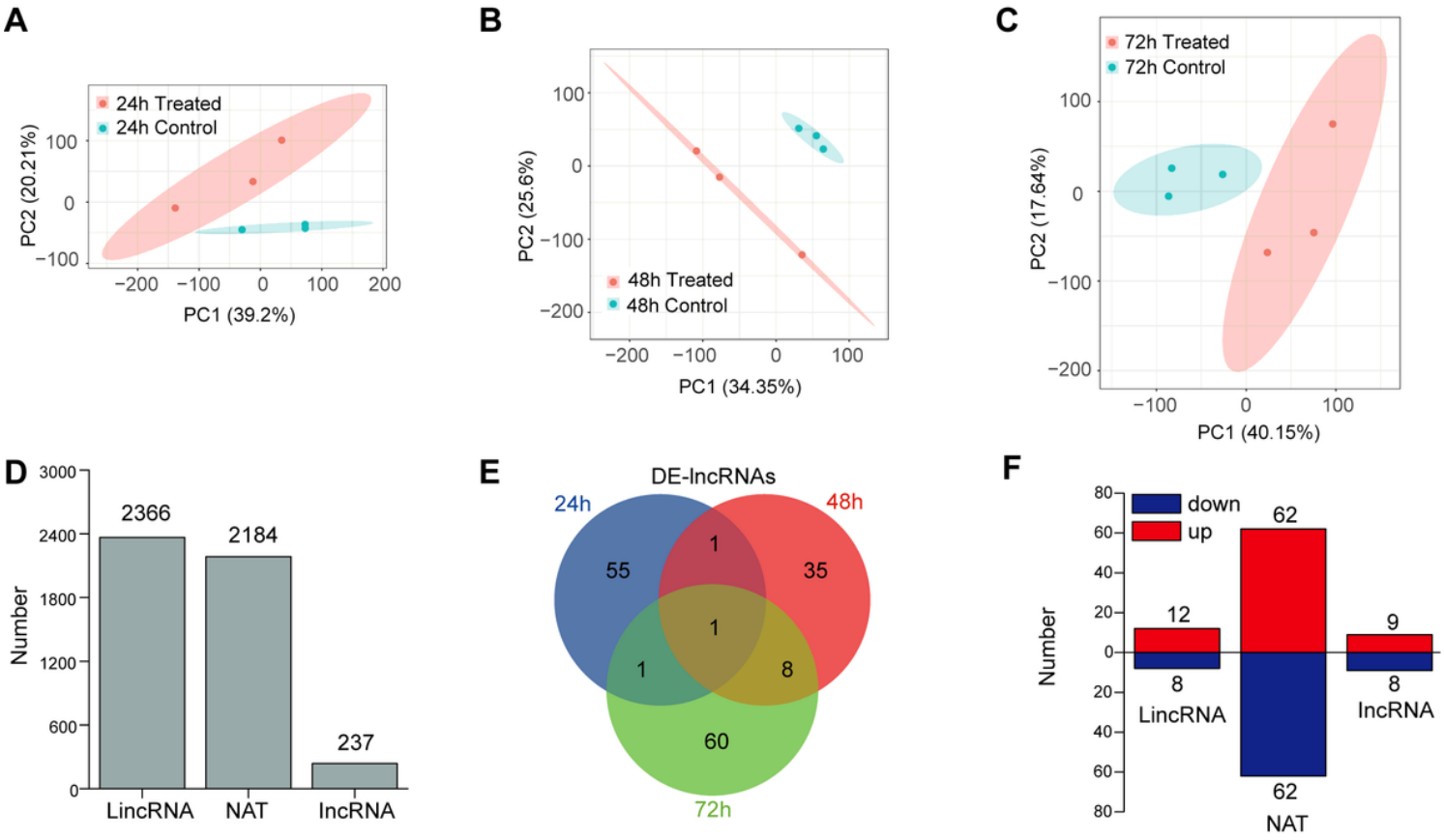

Figure 1

Overview of blast fungus-responsive lncRNAs in rice A-C. Principal Components analysis (PCA) of mRNA-sequencing data among different treatments. Control, mock treatment. Treated, M. oryzae treatment. D. Total numbers of identified lncRNAs in rice. LincRNA, long intergenic non-coding RNA. NAT, nature antisense transcripts. lncRNA, intronic RNA. E. A venn diagram showing differentially expressed (DE)

lncRNAs 24h, 48h and 72h after M. oryzae treatment. Foldchange > 2, p value < 0.05. F. Numbers of up- or down-regulated lncRNAs after blast fungus treatment.

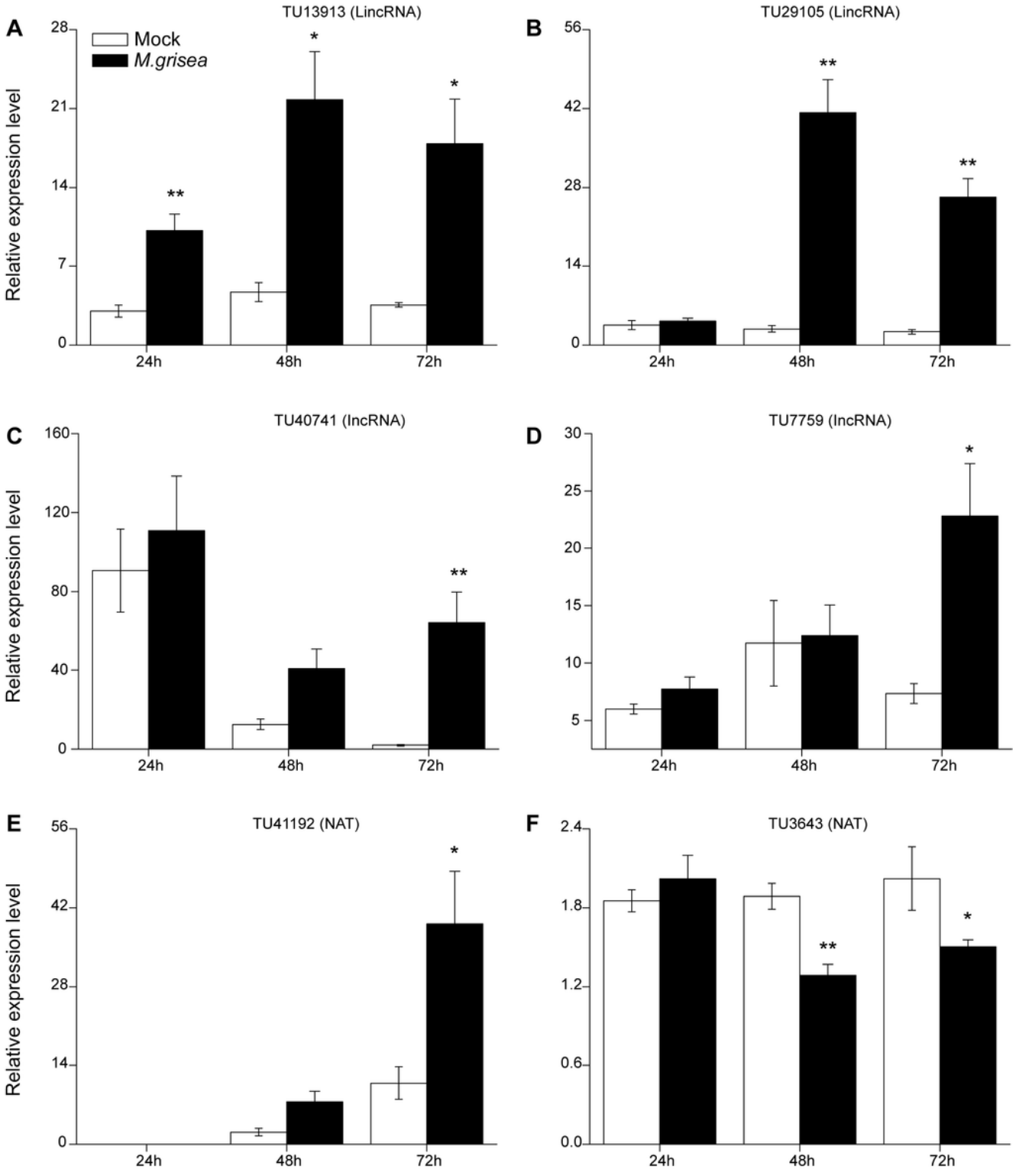

Figure 2

Confirmation of the transcriptional levels of DE-lncRNAs by RT-PCR Mean transcriptional abundance (±SE, n = 5-8) of lincRNAs (A, B), incRNAs (C, D) and NATs (E, F) in M. oryzae-treated samples and control

samples. Asterisks indicate significant differences in M. oryzae-treated samples compared with control samples (*, P < 0.05; **, P < 0.01; Student's t test).

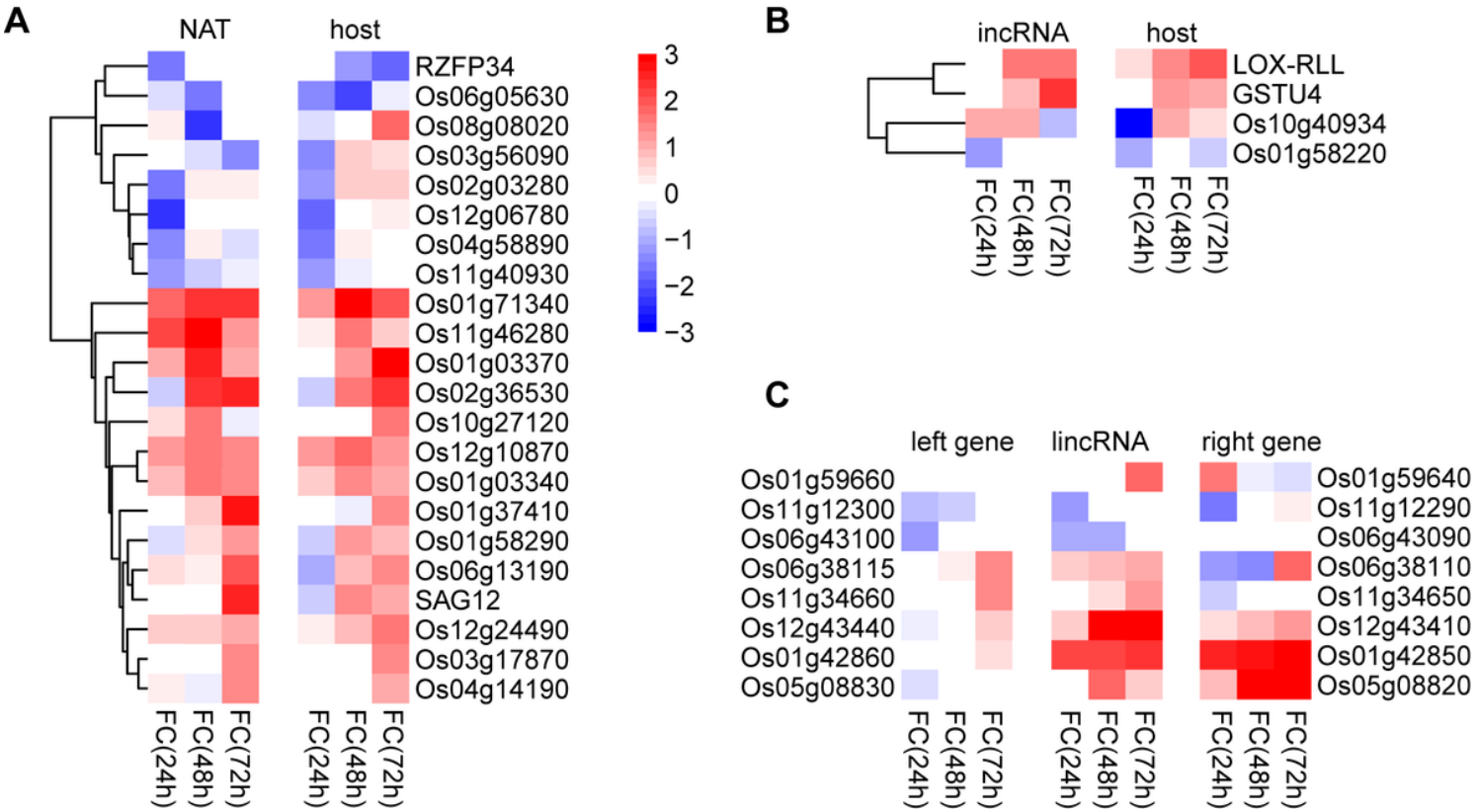

Figure 3

Expression pattern of blast fungus-responsive lncRNAs and their host or neighboring genes Heatmap representing the transcript abundance fold change (FC) of NAT (A), lncRNA (B) and their host gene in M. oryzae-treated samples compared with control samples. (C). Heatmap representing the transcript abundance fold change of lincRNA and its neighboring gene in M. oryzae-treated samples compared with control samples. U, up-regulated; D, down-regulated.

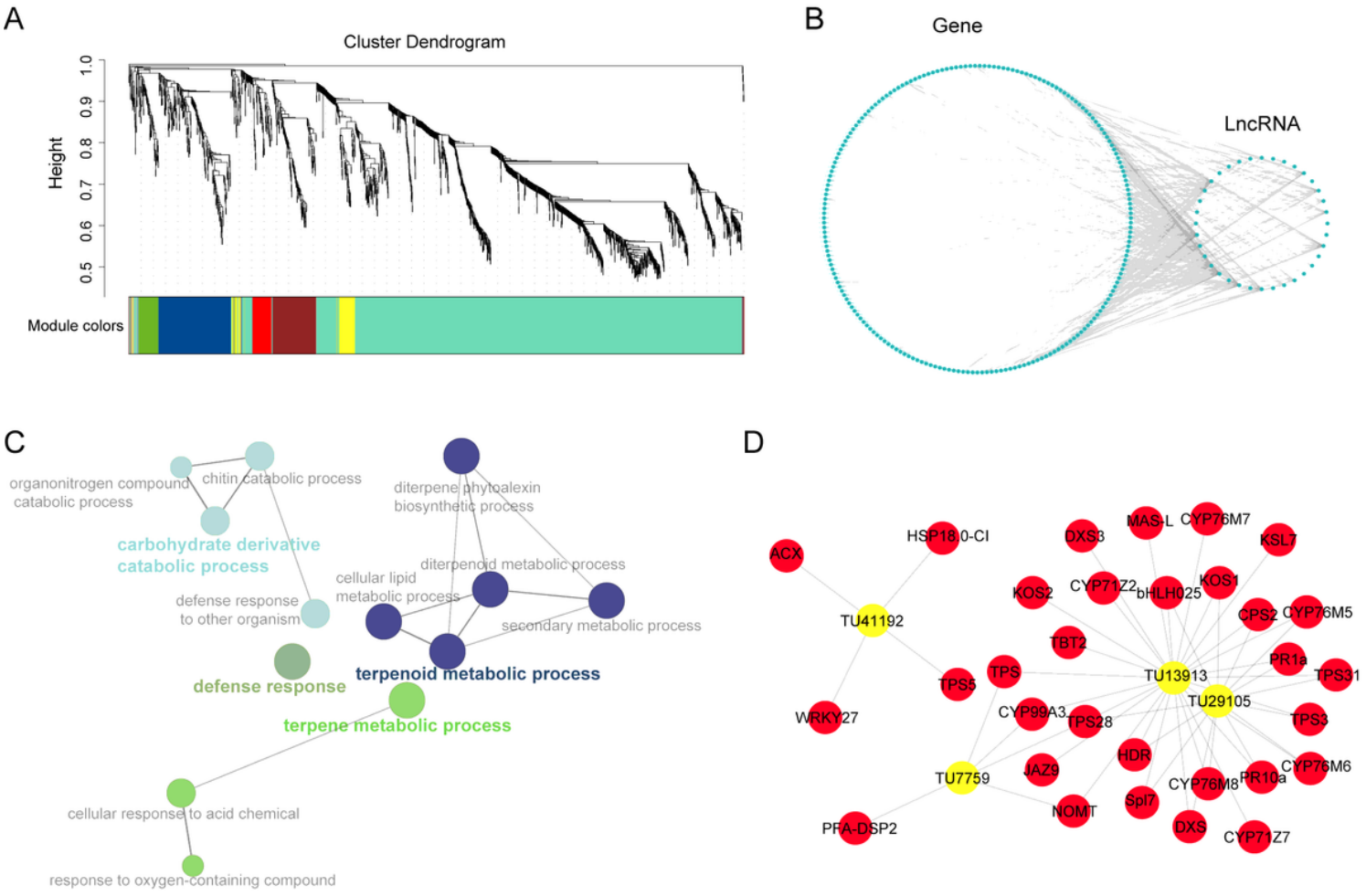

Figure 4

Co-expression analysis blast fungus-responsive genes and lncRNAs and GO enrichments of genes associated with lncRNAs A. Weighted correlation network analysis (WGCNA) of deferentially expressed (DE) lncRNAs and mRNAs. For DE-genes, we used the cutoff: foldchange > 2, Adjust p value < 0.05. Colors represent modules generated by WGCNA. B. Network of module turquoise on the basis of WGCNA analysis. We used the cutoff: weight > 0.4. C. Gene Ontology (GO) enrichment analysis of genes selected in fig. 4B. The size of character represents the significance of the GO; Color of the bubble represents GO group. D. Network plot of correlation between four DE-lncRNAs and known pathogen resistance-related genes. The information of these blast pathogen resistance-related genes were listed in Table. S2.

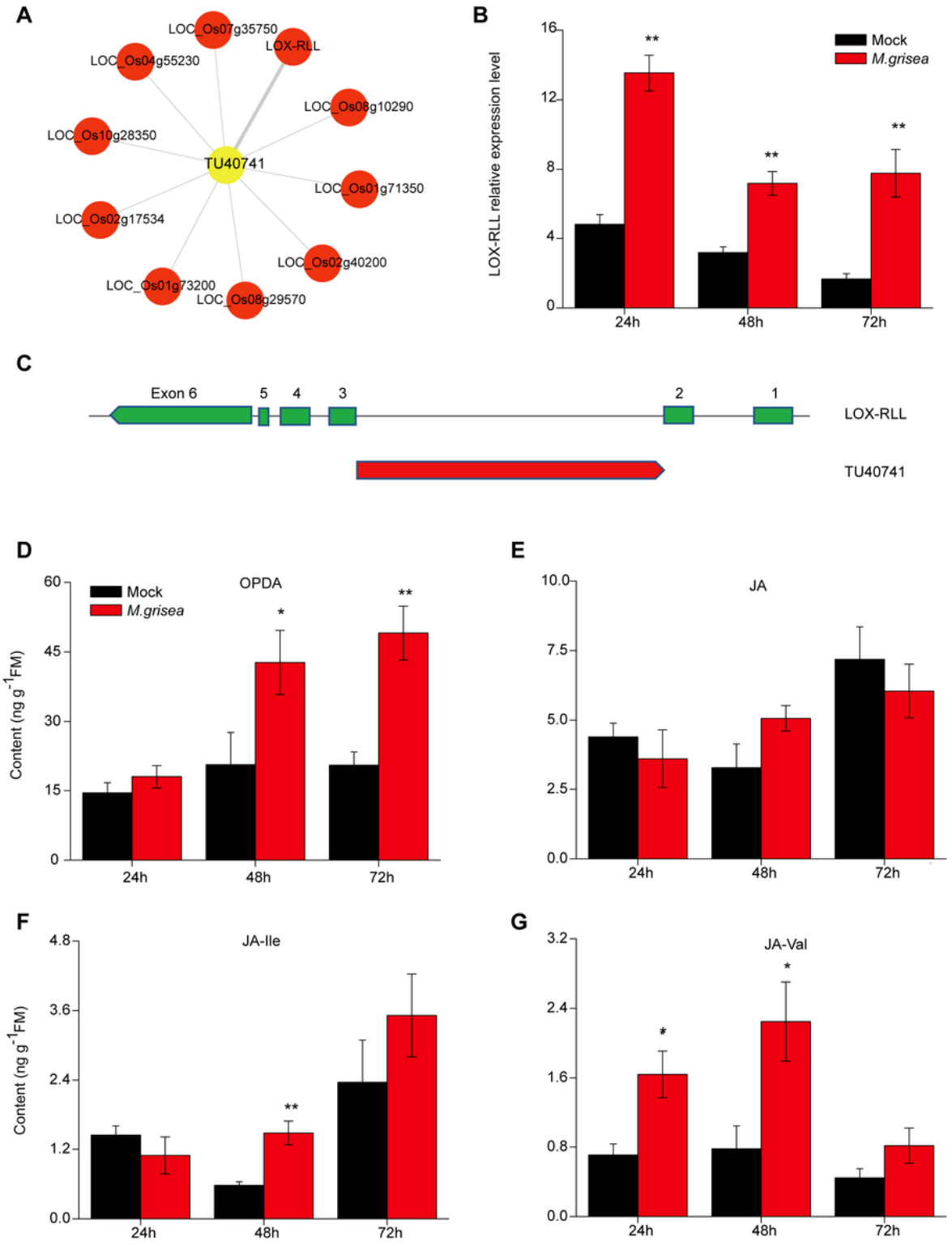

Figure 5

An intronic RNA is derived from jasmonate biosynthetic gene A. Network plot of correlation between a DE-incRNA and co-expressed genes based on WGCNA analysis. LOX-RLL, herbivore induced lipoxygenase. B. Mean transcriptional abundance (±SE, n = 5-8) of LOX-RLL in M. oryzae-treated samples and control samples. Asterisks indicate significant differences in M. oryzae-treated samples compared with control samples (**, P < 0.01; Student's t test). C. Schematic diagram of LOX-RLL and its intron-derived

transcripts. The six exons of LOX-RLL were highlighted by green. The intronic RNA was derived between exon 2 and exon 3, which has a opposite direction compared with LOX-RLL transcripts. Mean levels (±SE, n = 5-8) of OPDA (D), JA (E), JA-Ile (F) and JA-Val (G) in M. oryzae-treated samples and control samples. Asterisks indicate significant differences in M. oryzae-treated samples compared with control samples (**, P < 0.01; Student's t test).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS3.docx
- TableS2.docx
- TableS1.docx
- MaterialsandMethods.docx